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Utility of Pooled Urine Specimens for Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in Men Attending Public Sexually Transmitted Infection Clinics in Mumbai, India, by PCR

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Pooling urogenital specimens for the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by nucleic acid amplification tests is an attractive alternative to individual testing. As pooling can reduce the costs of testing as well as labor, it has been advocated for use in resource-poor settings. However, it has neither been widely adopted nor evaluated for use in developing countries. We evaluated the practical use of pooling first-catch urine (FCU) specimens for the detection of *C. trachomatis* and *N. gonorrhoeae* from 690 men in Mumbai, India, by PCR. FCU, urethral smears, and swabs were collected from men seen at two sexually transmitted infection (STI) clinics. All laboratory testing was done at the Lokmanya Tilak General Hospital. Gram stain smears and culture isolation for *N. gonorrhoeae* were performed. Each FCU was tested individually and in pools using the Roche Amplicor PCR for *C. trachomatis* and *N. gonorrhoeae* with an internal control for inhibition. Specimen pools consisted of aliquots from five consecutively processed FCUs combined into an amplification tube. An optical density reading of ≥0.20 indicated a pool for which subsequent testing of individual samples was required. Prevalence by PCR on single specimens was 2.2% (15/690) for *C. trachomatis* and 5.4% (37/690) for *N. gonorrhoeae*. Compared to individual FCU results, pooling for *C. trachomatis* and *N. gonorrhoeae* had an overall sensitivity of 96.1% (50/52). Specificity was 96.5% (83/86) in that three pools required single testing that failed to identify a positive specimen. Pooling missed two positive specimens, decreased the inhibition rate, and saved 50.3% of reagent costs. In this resource-limited setting, the use of pooling to detect *C. trachomatis* and *N. gonorrhoeae* by PCR proved to be a simple, accurate, and cost-effective procedure compared to individual testing.

Nucleic acid amplification tests (NAATs) for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are highly sensitive and specific. Commercially available tests include PCR, Amplicor (PCR; Roche Molecular Systems, Branchburg, N.J.), strand displacement amplification, BDProbeTec (SDA; Becton Dickinson Co., Sparks, Md.), and transcription-mediated amplification, COMBO2 (TMA; Gen-Probe Inc., San Diego, Calif.) (16, 17, 23, 24). Despite their utility, NAATs are expensive and the high cost has been a deterrent to their routine use in many laboratories. Lisby et al. evaluated pooling endocervical and urethral samples for *C. trachomatis* detection (13) as a cost-saving strategy. The concept of pooling involves screening multiple samples in groups. If any pool is positive, then specimens comprising the pool are tested individually to identify the true positive and negative specimens. Because commercially available NAATs are not approved by the Food and Drug Administration for use with pooled samples, this procedure must be validated prior to its clinical use. Several pooling studies have been performed using PCR and LCR (Abbott Laboratories, Abbott Park, Ill.; product discontinued) and have focused mainly on *C. trachomatis* detection (3, 4, 8, 9, 11, 19, 20). These studies found the correlation of *C. trachomatis* results with individually tested specimens to be excellent. Pooling urine or swabs for *C. trachomatis* detection of samples from low-prevalence (<5%) populations can reduce the cost of testing by saving reagents and technician labor. These decreased costs have also allowed laboratories to achieve a significant increase in specimen loads. In the United States, approximately 12% of laboratories perform pooling of clinical specimens for *C. trachomatis* (25). Although pooling has been advocated for use in resource-limited countries for these reasons, its utility in such settings has not been evaluated.

A major limitation to pooling urogenital specimens for NAATs has been the added time required for all the retesting. First, a repeat test must be performed to identify the individual positive patient in the pool. Then, a repeat test may be performed to confirm the positive specimen. The latter retest is optional and only done if laboratories are following the 2002 Centers for Disease Control and Prevention guidelines' suggestions to confirm positive NAATs (2). If all repeat tests are not completed within the same day, then an increase in turnaround time occurs.

Other areas that require investigation include determining

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whether pooled samples can be used for *N. gonorrhoeae* detection. It is also unclear whether pooling increases the likelihood of inhibition. NAATs can be affected by inhibitors (14, 22). Although decreased sensitivity of NAATs with pooled samples has not been reported, internal controls (IC) have not been systematically employed to identify the role of inhibitors.

We evaluated the practical use of testing pooled first-catch urine (FCU) specimens from men in Mumbai, India, as an alternative to individual specimen testing for the detection of *C. trachomatis* and *N. gonorrhoeae* by Amplicor PCR. On a subset of men who had urethral discharge, PCR results for *N. gonorrhoeae* were compared to the reference standard of culture isolation and Gram-stained smears.

TABLE 1. Determination of PCR cutoff value for retesting of pools

Pool cutoff (OD reading)	*C. trachomatis*		*N. gonorrhoeae*	
	No. of pools[a]	No. of true-positive pools	No. of pools[a]	No. of true-positive pools
<0.075	115	0	98	0
≥0.075	23	13	40	30
≥0.100	15	13	34	30
≥0.150	15	13	32	30
≥0.200	15[b]	13	31[c]	30
≥0.250	15	13	31	30

[a] The total number of pools was 138.
[b] There were 13 true-positive and 2 false-positive CT pools.
[c] There were 30 true-positive NG pools and 1 false-positive NG pool.

MATERIALS AND METHODS

Patient population. Samples used in this study were obtained from men enrolled in a randomized controlled trial of a behavioral intervention to reduce human immunodeficiency virus and STI incidence. The parent study was approved by the Committee on Human Research at the University of California San Francisco and the local Institutional Review Board, which is covered by an National Institutes of Health Federal Wide Assurance. Informed consent was obtained from all patients. Subjects were recruited from men seeking evaluation of STI symptoms or human immunodeficiency virus testing at two public health clinics: the Sion Lokmanya Tilak General Hospital and the Mumbai Municipal Corporation STI Clinic in Mumbai, India. At the time this substudy was conducted, a total of 1,810 men had been enrolled. As part of the baseline evaluation, all participants submitted FCU specimens for screening of *C. trachomatis* and *N. gonorrhoeae* by PCR and swabs of urethral discharge were obtained from a limited number of symptomatic men. We evaluated 690 consecutive FCU specimens.

Specimen collection. All subjects provided 25 to 30 ml of FCU in a sterile collection cup. Swabs of urethral discharge were streaked onto Thayer-Martin plates and onto slides for Gram stain evaluation. *N. gonorrhoeae* plates were placed in a candle jar at 36°C. Urethral smears were air dried, heat fixed, and Gram stained on site and then placed in slide boxes at room temperature. Prior to processing, FCU specimens were held at 4°C. All specimens were transported to the Lokmanya Tilak General Hospital laboratory at the end of each day, where final testing was performed.

Direct Gram stain. A positive smear for gonorrhea had gram-negative diplococci with typical morphology identified within or closely associated with polymorphonuclear leukocytes.

***N. gonorrhoeae* isolation.** Inoculated Thayer-Martin plates were incubated in candle jars at 36°C for 48 h (10). Presumptive *N. gonorrhoeae* colonies were Gram stained, oxidase tested, and subcultured onto chocolate agar. Pure cultures were confirmed by Gonochek II (EY Laboratories, San Mateo, Calif.).

PCR specimen processing. Specimens were processed, held at 4°C, and tested within 7 days of collection. Individual urine specimens were prepared up to the point of preamplification and stored until tested in batches. FCUs were swirled in the specimen cup for 10 s. A 500-μl aliquot was placed into a Sarstedt tube along with 500 μl of urine wash buffer. The tube was vortexed and incubated at 37°C for 15 min. FCUs were centrifuged at ≥12,500 × *g* for 5 min. The supernatant was discarded and the pellet resuspended in 250 μl of lysis buffer. After a 15-min incubation at room temperature, 250 μl of specimen diluent was added and vortexed and then centrifuged at ≥12,500 × *g* for 10 min. These processed specimens were ready for amplification. A pool was made by placing 10 μl of each processed specimen into an amplification vial to create a 5× pool, for a total volume of 50 μl.

PCR assay. Individual and pooled processed specimens were tested on the same run in accordance with the manufacturer's specifications (16, 24). Briefly, 50-μl volumes of controls, individual specimens, and pooled specimens were placed into reaction tubes along with the master mix containing the *C. trachomatis*, *N. gonorrhoeae*, and IC primers. The *C. trachomatis* primers consisted of a 207-nucleotide sequence within the cryptic plasmid of *C. trachomatis*. The *N. gonorrhoeae* primers consisted of a 201-nucleotide sequence within the putative cytosine DNA methyltransferase gene of *N. gonorrhoeae*. The IC was a DNA plasmid with primer binding regions identical to those of the *C. trachomatis* target sequence. Samples were thermocycled in a GeneAmp PCR System 9600 (approximately time = 70 min). The program ran 1 cycle for uracil-*N*-glycosylase activation and denaturation; 35 cycles for target denaturation, primer annealing, and amplicon extension; and a final hold program at 72°C. After thermocycling, amplicons were denatured and hybridized to oligonucleotide probe-specific targets. Three separate detections (*C. trachomatis*, *N. gonorrhoeae*, and IC) were done using avidin-horseradish peroxidase as the conjugate and tetramethylbenzidine as the substrate. The plates were read at 450 nm.

PCR cutoff. A positive result for individual specimen was defined as an optical density (OD) reading of ≥2.0 for *C. trachomatis* and ≥3.5 for *N. gonorrhoeae*. Equivocal results had OD readings of 0.2 to the cutoff OD for either *C. trachomatis* or *N. gonorrhoeae*. *N. gonorrhoeae* equivocal specimens were repeat tested two times. If two out of three OD readings for *N. gonorrhoeae* were above ≥2.0, the specimen was identified as positive. Specimens with an OD of <0.2 on the internal control were considered to be inhibited. As recommended by Kacena et al., an OD cutoff was established for pooled specimens (8). To determine the positive cutoff for a pooled specimen, individual OD readings were compared to pooled specimen OD readings. An OD reading that identified the highest number of positives and had a minimum number of pool retests was selected (Table 1). We determined that an OD cutoff of ≥0.200 resulted in the best performance profile.

Calculation for sensitivity and specificity. A true-positive pool had an OD reading of ≥0.200 that contained at least one or more specimens with individual *C. trachomatis*- or *N. gonorrhoeae*-positive results. A false-negative pool failed to identify pools that contained individual positive specimens. A pool with an OD of ≥0.200 that upon retest of individual specimens did not yield any positive *C. trachomatis* or *N. gonorrhoeae* result was defined as a false-positive pool. Because of limited resources, discrepant analysis of these false-positive pools was not investigated further.

RESULTS

The prevalence of *C. trachomatis* and *N. gonorrhoeae* by PCR in this population was 2.2% (15/690) and 5.4% (37/690), respectively. Table 2 shows the performance of pooling specimens compared to individual FCU testing. A total of 138 pools were tested, of which 15 and 31 were positive for *C. trachomatis* and *N. gonorrhoeae*, respectively. Five were positive for both *C. trachomatis* and *N. gonorrhoeae*. We found that pooling for *C. trachomatis* and *N. gonorrhoeae* had an overall sensitivity of 96.1% (50/52) compared to individual results. Specificity was 96.5% (83/86). Pooling missed two positive specimens, one *C. trachomatis* with an OD reading of 2.146 and one *N. gonorrhoeae* with an OD reading of 3.120 (Gram stain or culture was not performed on this asymptomatic patient). Neither missed positives showed inhibition with the internal control. There were three (two *C. trachomatis* and one *N. gonorrhoeae*) presumptive positives pools that were determined to be false positives upon retesting the individual specimens. The percentage of specimens that were inhibited decreased from 1.7 to 0% with pooled FCUs.

On the basis of testing for *N. gonorrhoeae*, pooling resulted

TABLE 2. Accuracy of pooling urine specimens for detection of *C. trachomatis* and *N. gonorrhoeae* by PCR

Parameter	*C. trachomatis*	*N. gonorrhoeae*	Combined
No. of individual specimens	690	690	
No. of individual specimens positive (%)	15 (2.2)	37 (5.4)	
No. of individual specimens inhibited (%)	12 (1.7)	12 (1.7)	
No. of pools made	138	138	
No. of presumptive positive pools	15	31	
No. of true-positive pools (%)	13 (9.4)[a]	30 (21.7)[b]	
Sensitivity of pooling (%)	13/14 (92.9)	30/31 (96.8)	
Specificity of pooling (%)	122/124 (98.4)	106/107 (99.1)	
Sensitivity of pooling as single testing (%)	14/15 (93.3)	36/37 (97.3)	
No. of inhibited pools	0	0	
Total no. of tests performed[c]	213	293	343
Total no. of tests saved	477	397	347
Total savings in tests (%)	69.1	57.5	50.3

[a] One pool had two positive CT specimens.
[b] Four pools had two positive NG specimens, and one pool had three positive NG specimens.
[c] Number of tests performed = 138 pools + (number of presumptive positive pools × 5).

in a reagent cost savings of 57.5% (Table 2). Greater savings were seen with testing for *C. trachomatis* (69.1%). Testing for *C. trachomatis* and *N. gonorrhoeae* simultaneously (the usual standard) resulted in a reagent cost savings of 50.3% by pooling specimens.

Only specimens from 31 patients were Gram stained and cultured for *N. gonorrhoeae*, of which 2 were identified as true negatives. Compared to Gram-stained smear- and culture-positive or Gram-stain smear- and PCR-positive specimens, PCR of pooled specimens was highly sensitive 93.1% (27/29). Culture missed 11 *N. gonorrhoeae*-positive specimens that pooling identified. Thus, sensitivity for culture was only 62.1% (18/29). We detected more *N. gonorrhoeae* positives by Gram stain than by culture isolation.

DISCUSSION

Because of the high cost, there are only a limited number of laboratories using NAATs for the detection of urogenital chlamydia infection in India. Most laboratories and clinics do not test for *C. trachomatis*. Among those that do; the majority use either direct fluorescent-antibody assays or enzyme immunoassays or even serologic tests because they are more affordable. Clearly, these tests lack both sensitivity and specificity (12, 18); however, they continue to be used in India for studies of STI prevalence, primarily among women (1, 5, 15). On the basis of these studies, the prevalence of *C. trachomatis* among asymptomatic women has consistently been reported as being low. Recently, Singh et al. found that 28% of symptomatic young women in New Delhi had *C. trachomatis* infections when tested with a homebrew PCR (21). Whereas in a population-based study using Amplicor PCR, the prevalence of genital chlamydial infection was reported to be 1.1% in men and women (6).

In contrast to studies among women, there are relatively few studies on the prevalence of *C. trachomatis* in the male Indian population. In our study, we identified a prevalence of 2.2% for *C. trachomatis* and 5.4% for *N. gonorrhoeae* in men by PCR testing. Although these numbers are low, similar results were found among slum dwellers in Chennai using PCR testing (D. D. Celentano, Int. Conf. AIDS, abstr. C11007, 2002). As population rates of *C. trachomatis* are low, pooling of specimens is idea to obtain significant cost savings.

The Amplicor PCR is configured so that *C. trachomatis* or *N. gonorrhoeae* can be tested alone or simultaneously. If combined testing is done, then there is an overall reagent cost savings of 50.3% as a result of pooling specimens. Rather than testing 690 specimens, we tested 343 (138 pools and 205 individuals specimens from positive pools). The use of the processed sample as the specimen to be pooled allows a cost savings evaluation that is truly based on the number of tests run. When prevalence rates exceed 10%, the benefits of pooling become less attractive due to the increased cost of retesting pools (11).

Compared to individual FCU results, pooling for *C. trachomatis* and *N. gonorrhoeae* had a combined sensitivity of 96.1% (50/52). Specificity was 96.5% (83/86). These results are similar to previous PCR pooling studies (13, 19, 20). However, Morre et al. found slightly better sensitivity and specificity (100% for both) with pools of five FCUs and no observed inhibition in men (19). Their protocol involved pooling unprocessed specimens. We had 93.3% sensitivity and 98.4% specificity with *C. trachomatis* pooling. In our study, we pooled processed specimens to allow a faster turnaround time in identifying the individual positive specimens within a pool. Laboratories that routinely perform pooling will likely use processed specimens.

Pooling of specimens for detection of *N. gonorrhoeae* is less commonly used. There is only one published study, by Kacena et al., using LCR testing of female urine samples for *N. gonorrhoeae* detection (7). Although LCR is no longer available, pooling of samples for *N. gonorrhoeae* detection using other NAATs theoretically should work. When we compared pooled *N. gonorrhoeae* testing with culture isolation and Gram-stained smears, the sensitivity of pooling was 93.1%. It is known that PCR is more sensitive than culture (16). In this study, Gram-stained smears identified more cases of *N. gonorrhoeae* than culture. There were 11 specimens that were smear and PCR positive but culture negative. The lower performance of culture is probably due to difficulties in maintaining an optimum culture system and preparation of quality media in our setting.

There have been some concerns that pooling might increase NAAT inhibition. However, we found the opposite; pooling specimens decreased the inhibition rate from 1.7% (12/690) to 0% (0/138). The observed decrease in inhibition may be due to dilution of inhibitors in pooled samples. The two positive specimens that were missed by pooling were not inhibited. Our rate of inhibition in male FCUs was similar to rates seen in previous PCR clinical evaluations (22, 24).

In conclusion, we found that the use of pooling to detect *C. trachomatis* and *N. gonorrhoeae* by PCR is a simple, accurate, and cost-effective procedure compared to individual testing of men attending public STI clinics in Mumbai, India. Once validated in the laboratory, this procedure can be feasible in resource-limited settings. In this setting, further evaluation in samples from women may be warranted since women are the most likely to be screened and differential inhibition rates are seen with female urine.

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